Journal of Medical Virology 49:145–154 (1996)

Characterization of a New Genotype II Hepatitis Delta Virus From Taiwan

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Three genotypes of HDV, which may be associated with different clinical pictures and epidemiological patterns, have been identified. In contrast to Type I and Type III HDV, both of which have multiple isolates, Type II HDV so far includes only a single isolate (Japan-1) from a low prevalence area (Japan). Recently, Type II has been reported to be the predominant genotype in Taiwan, which is also a low prevalence area, and is associated with less aggresive disease than Type I. However, the sequence and structure of these viruses have not been characterized. The complete characterization of a second member (Taiwan-3 isolate) of the Type II HDV from Taiwan is reported. These two Type II HDV isolates (Taiwan-3 and Japan-1) have 93.8% nucleotide homology and 89.3% amino acid homology, respectively. These shared sequences establish the common characteristics of Type II viruses. Sequence comparisons of various HDV genotypes show that the autocatalytic region of the RNA is relatively conserved between Type I and Type II (88.5–95.6% homology) but is significantly divergent in Type III (76.8–80.3% homology). The hypervariable region (nucleotides 1602–658) of RNA, however, is heterogeneous (64.9–73.0%) among all three genotypes. The delta antigen sequence is also very heterogeneous (64.9–73.0%). Most strikingly, the C-terminal sequence (19 amino acids) of the large delta antigen is almost completely different in each of the three genotypes. The heterogeneity in this region of three HDV genotypes may be a basis for their different biological properties, and the nucleotide sequences of this region can be used to differentiate the different genotypes of HDV. The consensus sequence in the four previously identified conserved domains of HDV RNA is defined more precisely.

KEY WORDS: hepatitis delta virus, PCR, sequence analysis, genotypes

INTRODUCTION

Hepatitis delta virus (HDV) is a defective virus, containing a single-stranded circular RNA genome of 1.7 kilobases (kb) [Wang et al., 1986; Makino et al., 1987], a delta antigen, and an envelope consisting of hepatitis B surface antigen [Rizzetto et al., 1980]. Thus, simultaneous presence of hepatitis B virus (HBV) infection is a requisite for HDV transmission. HDV RNA has a unique autocatalytic cleavage and ligation activity [Wu and Lai, 1989; Kuo et al., 1988], and encodes at least one protein, hepatitis delta antigen (HDAg), from its antigenomic strand [Wang et al., 1986]. HDV infection is endemic in the Mediterranean region, the Middle East, and South America [Rizzetto 1983; Ponzetto et al., 1985; Hadler et al., 1991]. However, interestingly, the incidence of delta hepatitis is relatively low in Asia, despite the high prevalence rate of HBV infection and a high carrier rate of HBV in several regions, including Taiwan [Chen et al., 1984].

HDV has been found to be associated with fulminant hepatitis, acute hepatitis, chronic hepatitis, and cirrhosis [Govindarajan et al., 1984; Smedile et al., 1983]. The wide clinical spectrum implies a complex mechanism of pathogenesis [Rizzetto et al., 1980; Schlipkoter et al., 1990; Brunetto et al., 1991; Faure et al., 1991; Negro and Rizzetto, 1993]. Of note is that HDV infection in different geographic areas appears to differ in severity [Ponzetto et al., 1985; Rizzetto, 1983; Liaw et al., 1987; Govindarajan et al., 1984; Smedile et al., 1983; Faure et al., 1991; Negro and Rizzetto, 1993]. The differences in clinical manifestations of HDV infection may result from the possible genetic differences in the predominant HDVs prevalent in different geographic areas [Faure et al., 1991; Lee et al., 1992; Chao et al., 1991; Casey et al., 1993; Imezeki et al., 1991; Wu et al., 1995].

So far, three major genotypes of HDV have been identified [Casey et al., 1993]. Type I has been found worldwide including Taiwan [Casey et al., 1993; Chao et al., 1991].

Accepted for publication 21 February, 1996.

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Type III, which is usually associated with severe fulminant hepatitis [Casey et al., 1993], is limited to South America. Type II was found originally only in Japan [Imezeki et al., 1991], where HDV prevalence is low. Recently, Type II was found to be the predominant HDV genotype in the northern part of Taiwan [Wu et al., 1995]. Interestingly, it is associated less frequently than Type I with fulminant hepatitis during the acute stage; it also has a more favorable long-term clinical outcome than Type I [Wu et al., 1995]. However, so far only one HDV isolate (Japan-1) from Japan has been characterized [Casey et al., 1993; Imezeki et al., 1991]; and therefore, the characteristics of Type II HDV cannot be determined with certainty. The characteristics of the second Type II HDV are described.

MATERIALS AND METHODS

Preparation of Nucleic Acids

Serum was obtained from a 30-year-old male patient with chronic delta hepatitis. Biochemical test revealed Albumin/Globulin 4.7/3.0 g%, AST 57 U/L, ALT 112 U/L, total bilirubin 0.6 mg%. Radioimmunoassay (Abbott Laboratories, North Chicago, IL) revealed HBsAg (+), antidelta (+), HBeAg (−), antiHBe (+), and alpha-fetoprotein <3 ng/ml. Enzyme immunoassay (NANBA-SEC-96, General Biological Corp., Taiwan) revealed absence of antiHCV. Ultrasound examination showed normal liver parenchyma, surface, and size, normal gallbladder, and biliary tree. Liver biopsy showed mild chronic active hepatitis. Past history included sexual contacts with prostitutes. He had neither blood transfusion nor intravenous drug abuses before. RNA was extracted from serum by using a single-step method with acid guanidinium thiocyanate-phenol-chloroform [Chomczynski et al., 1987]. Briefly, serum was mixed with a denaturing solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sodium N-laurylsarcosine, and 0.1M β-mercaptoethanol. RNA was extracted with 0.2 M sodium acetate, pH 4, phenol and chloroform, and was precipitated with isopropanol. The RNA was dissolved in the diethyl pyrocarbonate-treated distilled water and stored at −70°C.

Synthesis of cDNA

RNA from 30 ul serum was used for cDNA synthesis. RNA was denatured at 94°C for 2 min and then chilled on ice. Reverse transcription was carried out at 42°C for 30 min in a 50 ul of buffer containing 5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 8.8, 0.1% Triton X-100, 1 mM each of the deoxyribonucleoside triphosphates, 0.5 units of ribonuclease inhibitor RNasin (Promega), 1 uM synthetic oligonucleotide primer (antisense primer), and 15 units of reverse transcriptase (Promega).

Amplification of cDNA by PCR

For polymerase chain reaction (PCR), one-fifth of the reaction mixture of reverse transcription as described above was mixed with 90 ul of a buffer containing 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 0.1% Triton X-100, 10 mM $MgCl_2$, 200 uM each of the deoxyribonucleoside triphosphates, 2.5 units of Taq polymerase (Promega), and 0.5 uM of a synthetic oligonucleotide primer (sense primer) and amplified by PCR according to the method of Saiki et al. [1988]. PCR was performed for 40 cycles, each cycle consisting of 95°C, 1 min; 40°C, 1 min; 72°C, 1 min. The sequences of the primers for reverse transcription and PCR are shown in Table I. The PCR products were analyzed by electrophoresis in 1.5% agarose gel, and the specific products were purified from the gel for cloning.

Cloning, Sequencing, and Analysis of Sequence

The specific PCR products were ligated with PT7 blue vector (Novagen) and transfected into DH5 α cell (BRL) for cloning. Sequencing was done using double-stranded DNA of plasmid clones. Dideoxy chain termination sequencing was undertaken using Sequenase version 2.0 sequencing kit (U.S. Biochemicals). The sequencing products were analyzed in 6% polyacrylamide gel containing 6 M urea. Sequence analysis was performed using DNASTAR program (DNASTAR Inc., WI). DNA sequences were aligned by the Wilbur and Lipman method [Wilbur et al., 1983] and amino acid sequences were aligned by the Needleman-Wunsch method using DNASTAR program (AALIGN V1.65; DNASTAR Inc., WI).

RESULTS

Five overlaping cDNA segments representing the entire HDV RNA were obtained (Fig. 1). At least five individual clones for each segment were sequenced. This RNA, designated Taiwan-3 isolate, totals 1676 nucleotides. The complete nucleotide sequence and amino acid sequence of the predicted delta antigen of the Taiwan-3 isolate (T3) are shown in Figures 2 and 3, respectively, and compared with the published sequences of HDVs derived from different geographic areas [Wang et al., 1986; Makino et al., 1987; Lee et al., 1992; Chao et al., 1991; Casey et al., 1993; Imezeki et al., 1991; Chao et al., 1990; Saldanha et al., 1990]. Pairwise comparisons of different HDV isolates showed that the Taiwan-3 HDV is similar to Japan-1 isolate (93.3% homology) whereas it is related only approximately 69–78% to Type I and Type III. Therefore, Taiwan-3 HDV belongs to genotype II. Based on the common characteristics of the two Type II HDV isolates, the extents of nucleotide and amino acid sequence homology between different HDV genotypes were compared and are shown in Tables II and III.

Previous studies based on the sequence divergence of different Type I HDV isolates divided the RNA genome into three regions, i.e., the hypervariable region (nucleotides 1602–658), the autocatalytic region (nucleotides 659–959), and the delta-antigen-coding region (nucleotides 960–1601) [Chao et al., 1994]. The availability of common features of Type II HDV allowed us to extend this analysis to include both Type II and Type III (Table III). The autocatalytic region is conserved between Type I and Type II (88.5–95.6% homology) but is significantly divergent in Type III (76.8–80.3% homology) (Table III). The most divergent region is the hypervariable region

TABLE I. Sequences of the Synthetic Primers Used in This Study*

Sense primer	Sequence	Homologous to nucleotides
87	CTTCGTCGGTGATCCTGCCTCT	1288–1309
120	ATGCCATGCCGACCCGAAGAGGAA	889–912
126	GGTCCTCAGTGCTCTTTACTCTT	1619–1640
LE17	ACCCACGGTCGGGTGATCCACCAGG	265–288
76	CTGCAGGGTCCGCGTTCCATCCTT	654–677
Antisense to primer	**Sequence**	**Complementary nucleotides**
88	CCAGCAGTCTCCTCTTTACAGA	1663–1642
138	TGTTCGCTGAAGGGGTCCTCTGGA	332–309
143	GACCATGCCGGCCATCAGGTA	701–681
214	CTCAGGGGAGGGTTCTCCGACA	1334–1313
77	ACTCACAGGTTTGCGTCTCGCGTC	941–918

*The numbering of nucleotides is according to Makino et al. [1987].

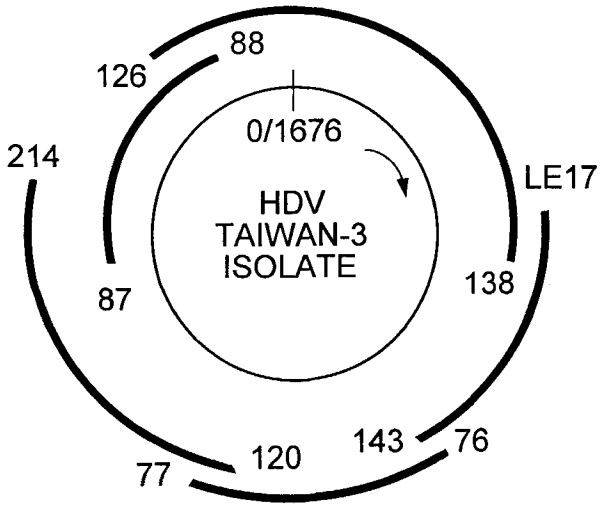

Fig. 1. Diagram of the Taiwan-3 HDV cDNA clones used for sequencing. The numerical names of the primers (Table I) used for PCR are indicated at both ends of these clones. The nucleotide 0/1676 is equivalent to nucleotide 0/1683 of the Southern California isolate of HDV [Makino et al., 1987]. The arrow represents the orientation of the genomic sense RNA of Taiwan-3 HDV.

(homology 61.5–70.5% among different genotypes) (Table III). The delta-antigen-coding region is also very divergent (68.5–81.3% homology) among different genotypes (Table III). The homology of amino acid sequences of delta antigen is 64.9–73.0% among all three genotypes.

Taiwan-3 HDV has one open reading frame (ORFs) capable of encoding more than 100 amino acids in each of genomic and antigenomic senses. The first one is from nucleotide 1430 to 434 of the genomic strand, and the second one is on the antigenomic sense from nucleotide 1593 to 949 (coding for HDAg). It is not clear whether the genomic strand ORF is functional or not. Microheterogeneity of nucleotide sequence and amino acid sequence of delta antigen were noted in several different places and are shown in parentheses in Figures 2 and 3.

DISCUSSION

Variation of the clinical spectrum of delta hepatitis from self-limiting hepatitis to fulminant hepatitis implies a complex mechanism of HDV pathogenesis [Rizzetto et al., 1980; Schlipkoter et al., 1990; Brunetto et al., 1991; Faure et al., 1991; Negro and Rizzetto, 1993; Casey et al., 1993]. The pathogenicity of HDV may be due to direct cytotoxicity of HDV RNA or delta antigen, the host immune reaction or the interaction between HDV and HBV. The genetic heterogeneity of HBV and HDV isolates may also account for at least part of the differences in the clinical picture of HDV infection [Rizzetto et al., 1980; Schlipkoter et al., 1990; Brunetto et al., 1991; Faure et al., 1991; Negro and Rizzetto, 1993; Casey et al., 1993].

HDV infection is endemic in the Mediterranean region, the Middle East, and South America. However, interestingly, the incidence of delta hepatitis is relatively low in the Asia, despite the high prevalence rate of HBV infection and a high carrier rate of HBV in several regions including Taiwan, where the HBV carrier rate in general population is 15% to 20%. HDV infection is relatively infrequent in asymptomatic HBV carriers (2–3%) and in patients with chronic liver disease (5–12%) [Chen et al., 1984; Liaw et al., 1987; Tai et al., 1989]. Previous reports [Rizzetto et al., 1983; Govindarajan et al., 1986; Hadler et al., 1984; Govindarajan et al., 1984] have shown that HDV superinfection might manifest as fulminant hepatic failure and usually results in a chronic aggressive course. The immediate mortality ranges from 2% to 20% in icteric acute HDV infection [Caredda et al., 1987]. About 41–60% of patients with chronic HDV infection develop cirrhosis [Rizzetto et al., 1983; Govindarajan et al., 1986]. In Taiwan, however, fulminant hepatic failure occurred less frequently [6% reported by Wu et al., in 1990] and cirrhosis occurred less frequently in chronic HDV infection (8.5%–11%) [Liaw et al., 1990, Wu et al., 1990]. HDV infection in different geographic areas appears to differ in severity [Ponzetto et al., 1985; Rizzetto, 1983; Liaw et al., 1987; Govindarajan et al.,

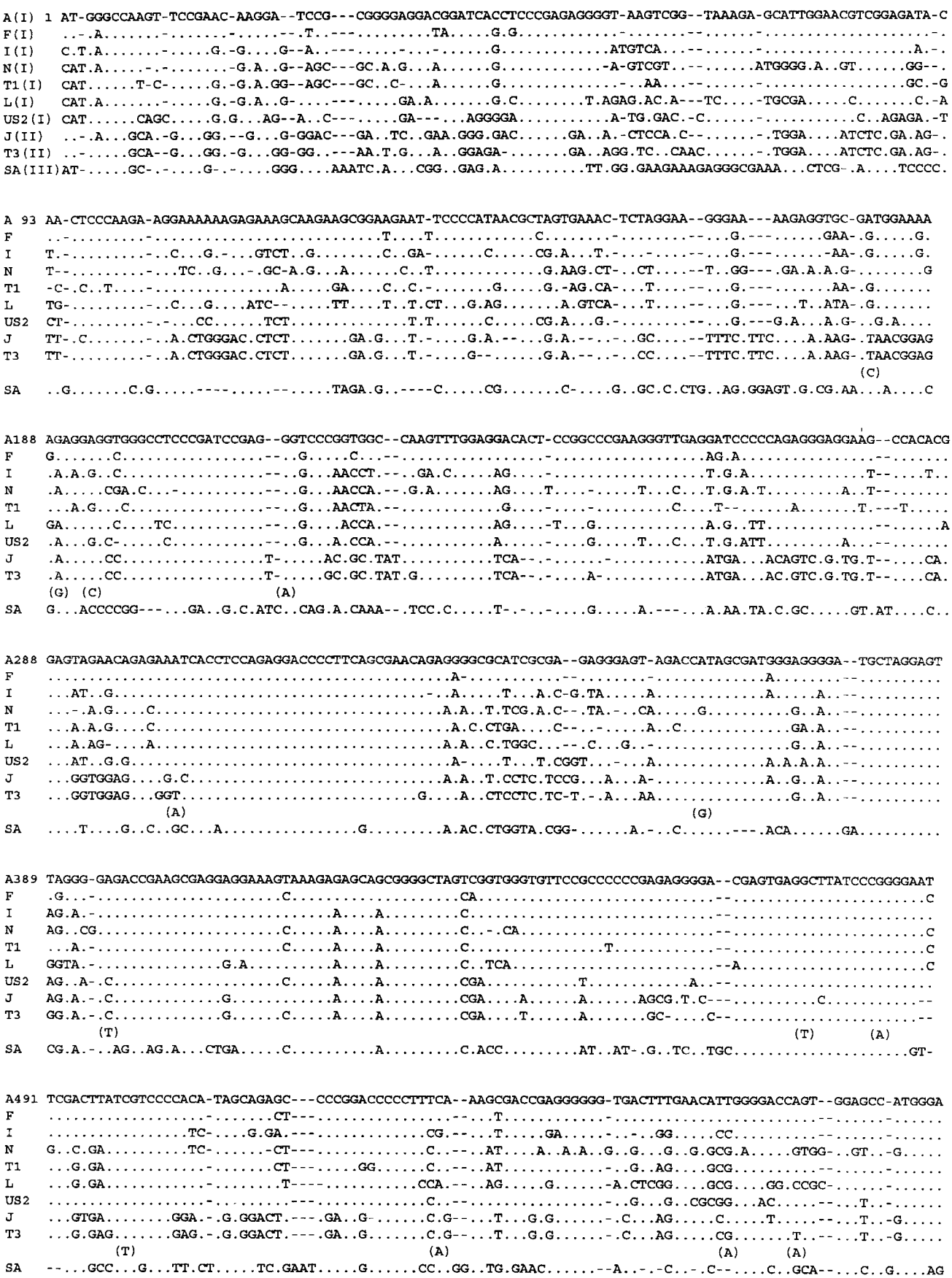

Figure 2.

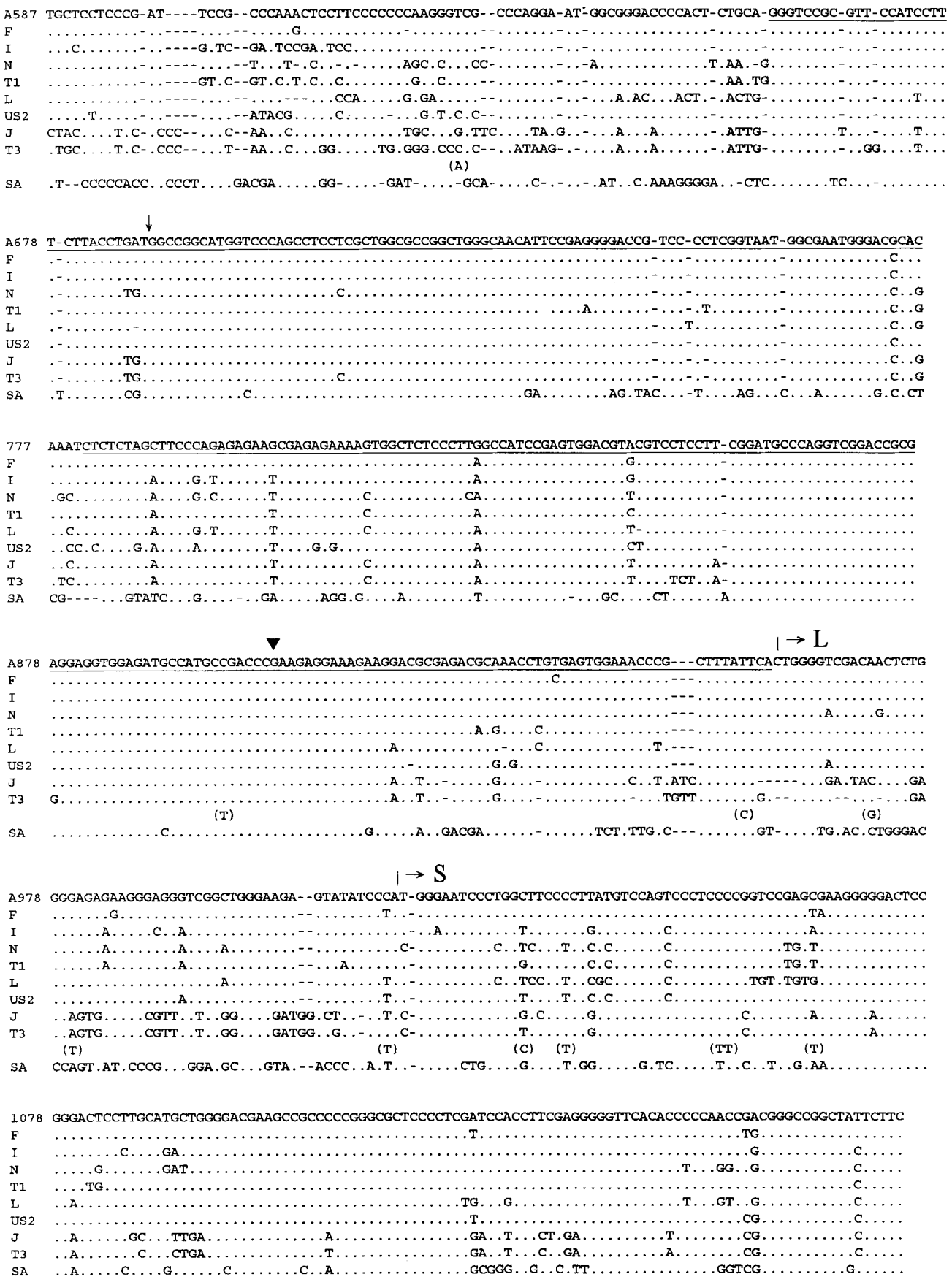

Figure 2. (cont.).

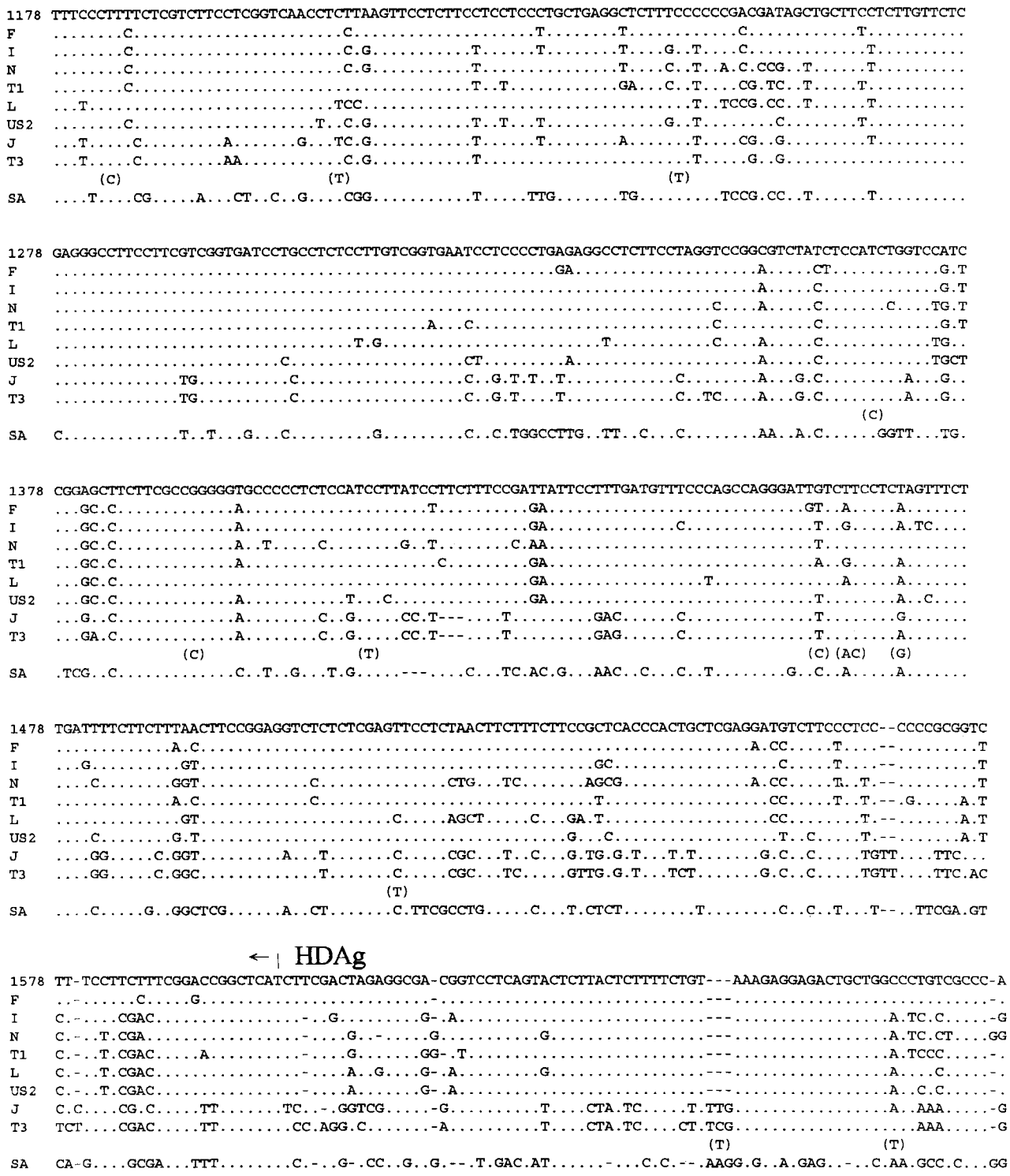

Fig. 2. Comparison of the cDNA sequences of the HDV RNA from Taiwan (T3) with published cDNA sequences. The published cDNA sequence of the Southern California HDV isolate (A) is used as the prototype sequence for comparison. The numbering of nucleotides is according to Makino et al. [1987]. The nucleotides, which show microheterogeneity, are indicated in parentheses. The antigenomic sequence which codes for the HDAg is not shown. However, the genomic site corresponding to the start codon and the orientation of the HDAg are indicated. The vertical lines indicate the termination sites for the small HDAg(S) and the large HDAg(L), respectively. The genomic autocatalytic cleavage site (688/689) is indicated by (↓). The site (903/904) corresponding to the cleavage site of the antigenomic RNA is indicated by (▼). The nucleotide sequences of autocatalytic region of the published isolates are underlined according to Chao et al. [1994]. The alphabetical letters represent the locations where the strains were isolated. Type I includes A: America-1 [Makino et al., 1987], F: France [Saldanha et al., 1990], I: Italy [Wang et al., 1986], N: Nauru [Chao et al., 1990], T1: Taiwan-1 [Chao et al., 1991], L: Lebanon [Lee et al., 1992], and US2: America-2 [Casey et al., 1993]; Type II includes J: Japan-1 [Imezeki et al., 1991] and T3: Taiwan-3 isolate of this study; Type III: SA: South America (Peru-1) [Casey et al., 1993].

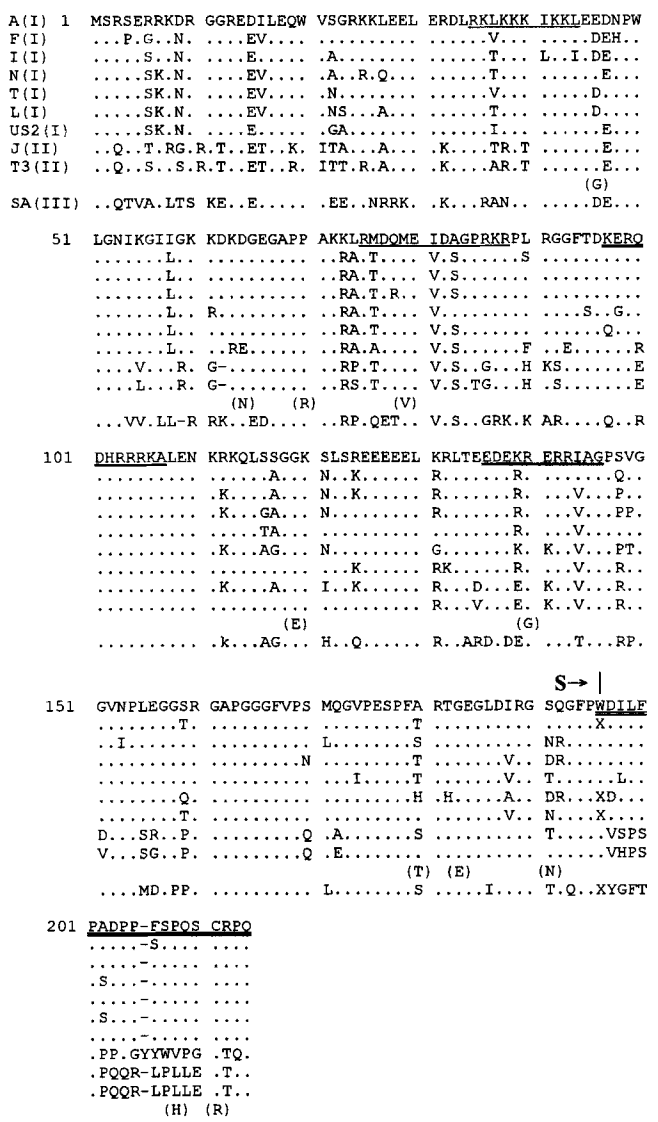

Fig. 3. Comparison of the amino acid sequences of HDAg between Taiwan-3 HDV and published sequences. The early termination codon on the antigenomic sense RNA for the small HDAg (S) of 195 amino acids is indicated by arrow. Type I includes A: America-1 [Makino et al., 1987], F: France [Saldanha et al., 1990], I: Italy [Wang et al., 1986], N: Nauru [Chao et al., 1990], T1: Taiwan-1 [Chao et al., 1991], L: Lebanon [Lee et al., 1992] and US: America-2 [Casey et al., 1993]; Type II includes J: Japan-1 [Imezeki et al., 1991] and T3: Taiwan-3 isolate of this study; Type III: SA: South America [Casey et al., 1993]. Nuclear localization signal [Chang et al., 1992] is marked by a thin line. RNA-binding domain [Lee et al., 1993] is marked by a thick line. Large delta antigen packaging signal [Chen et al., 1992] is doubly underlined.

1984; Smedile et al., 1983; Faure et al., 1991; Negro and Rizzetto, 1993]. A recent report suggests that Genotype II may be the predominant HDV genotype in Taiwan [Wu et al., 1995]. Genotype II was also found to be associated less frequently than genotype I with fulminant hepatitis at the acute stage; it also has a relatively more favorable long-term clinical outcome at the chronic stage than genotype I [Wu et al., 1995].

A vast majority of HDV isolates so far belong to Type I. Since these isolates were derived from many diverse geographical areas and associated with various clinical pictures, the Type I HDV may be a very heterogeneous group. In contrast, Type III isolates were restricted so far to South America [Casey et al., 1993] and were associated with severe fulminant hepatitis. Its uniform clinical picture and clustered geographical distribution suggest that Type III HDV has unique and homogeneous genetic properties. Curiously, Type II consists of only one HDV isolate from Japan [Casey et al., 1993; Imezeki et al., 1991], where HDV has low prevalence rate and low virulence [Ponzetto et al., 1985]. The isolation of Taiwan-3 HDV and the recent report of the predominance of Type II HDV in Taiwan [Wu et al., 1995] suggests the Type II HDV is associated with lower transmission and virulence. The complete characterization of this new HDV allowed the establishment of the common properties of Type II HDV. These two Type II HDV isolates share a higher degree of sequence homology than among most of the different isolates of Type I HDVs (Table II). The same degree of sequence homology between the two Type II HDVs is observed throughout the entire genome (Table III).

The availability of two complete Type II HDV sequences allowed the generalization of the properties of Type II viruses. The autocatalytic region (nucleotides 659–959) is very conserved between Type I and Type II (88.5–95.6% homology) but is significantly divergent in Type III (76.8–80.3% homology). The hypervariable region (nucleotides 1602–658), however, is heterogeneous in all HDV genotypes. The delta antigen sequence is also considerably heterogeneous (64.9–73.0%).

In previous studies [Chao et al., 1991; Lee et al., 1992; Chao et al., 1994], four conserved regions were recognized in Type I HDV isolates. Inclusion of the three genotypes for comparison defined further the highly conserved domains. The first conserved region is defined as from nt 688–733 (the numbering of nucleotides is according to Makino et al. [1987]), which corresponds to the sequence required for the autocatalytic cleavage activity of the genomic sense RNA. The second conserved region is defined as from nt 856–917, which is required for the autocleavage of the antigenomic sense HDV RNA. The third conserved region is defined as from nt 1196–1125. The fourth conserved region is defined as from nt 1268–1291, which corresponds to the middle domain of the open reading frame for HDAg, representing the RNA-binding domain [Lin et al., 1990].

There is marked heterogeneity at both the N-terminal and the C-terminal sequences of delta antigen. Most striking, the C-terminal sequence (19 amino acids) of the large-delta-antigen coding region is almost completely different in each of the three genotypes. This region has been shown to confer the ability of large delta antigen to suppress HDV RNA replication [Chao et al., 1990; Hwang and Lai, 1994] and to interact with the HBV surface antigen to form the HDV virus particles [Hwang and Lai, 1993]. The heterogeneity in this region of three HDV genotypes may be one of the bases for their different biological properties. It also suggests that the nucleotide sequences of this region can be used to differentiate

TABLE II. Nucleotide Homology (%) Among HDV Sequences in Different Geographic Areas*

	A	F	I	N	T1	L	US2	J	T3	SA
A	100	95.1	88.5	84.2	88.2	88.5	88.1	76.8	76.2	68.6
F		100	90.0	84.8	88.7	85.1	87.4	77.1	76.3	68.7
I			100	86.4	88.0	85.4	89.7	77.9	77.4	70.5
N				100	87.2	86.6	85.7	76.9	78.2	71.7
T1					100	86.8	87.5	76.9	77.1	68.2
L						100	84.8	76.2	76.4	68.8
US2							100	77.7	77.9	70.1
J								100	93.3	70.3
T3									100	69.2
SA										100

*I: America (A), France (F), Italy (I), Nauru (N), Taiwan-1 (T1), Lebanon (L), America-2 (US2). II: Japan-1 (J), Taiwan-3 (T3). III: South America (SA), (Peru-1).

TABLE III. Comparison of Nucleotide and Amino Acid Sequences Between Different HDV Genotypes*

	Type				
	I/I	I/II	I/III	II/II	II/III
Nucleotide homology (%)					
Complete sequence	84.2–95.1	76.2–78.2	68.2–71.7	93.3	69.2–70.3
Autocleave region	93.4–98.7	88.9–95.6	78.5–80.3	96.0	76.8–79.9
Delta-antigen-coding region	87.4–93.8	78.5–81.3	68.5–73.6	94.2	71.5–72.3
Hypervariable region	77.0–94.9	66.3–70.5	61.5–66.4	92.5	63.4–64.4
Amino acid homology (%)					
Delta antigen	84.1–91.6	68.8–73.0	64.9–68.3	89.3	64.9–68.3

*I: America (A), France (F), Italy (I), Nauru (N), Taiwan-1 (T1), Lebanon (L), America-2 (US2). II: Japan-1 (J), Taiwan-3 (T3). III: South America (SA) (Peru-1).

the different genotypes of HDV. Interestingly, the C-terminal 19-amino acids of HDAg are very conserved between the two Type II HDVs.

Similar to the other HDV isolates, there is a large number of sequence microheterogeneity in the Taiwan-3 isolate. As discussed previously, this is unlikely to be due to PCR errors [Chao et al., 1994]. The microheterogeneity may reflect viral heterogeneity, since all RNA viruses are considered to consist of a mixed population of viral RNA with minor sequence variation [Eigen and Biebricher, 1988].

In the Taiwan-3 isolate, there is one open reading frame (ORFs) capable of encoding more than 100 amino acids in each of genomic and antigenomic strand, the latter of which encodes the delta antigen. Similar to the Japan-1 isolate, the genomic strand of Taiwan-3 HDV has an open reading frame (ORF) from nt 1430–434 (Japan-1: nt 1437–182). However, comparison of the Taiwan-3 and the other published isolates (including all three genotypes) indicates that this ORF is not conserved. Among the three genotypes, there are two highly conserved regions in the HDAg-coding region, i.e., amino acids 101–115 which includes part of the RNA binding domain [Lee et al., 1993] and amino acids 160–169. The least conserved regions among the three genotypes are the N-termini of both forms of HDAg and the C-terminus of the large HDAg. It was suggested that the autocatalytic regions of genomic and antigenomic senses and several functional domains of delta antigen, e.g., nuclear localization signal [Chang et al., 1992], RNA-binding domain [Lee et al., 1993], and large delta antigen packaging signal [Chen et al., 1992] may play a role in the replication of HDV. However, comparison of sequences of the Taiwan-3 and the other isolates shows that there are some variations among these isolates in all these functional domains of delta antigen (Figures 2 and 3). Therefore, the nucleotide sequence requirement for these functions may not be very stringent. Furthermore, the base-paired seem structure which has been shown to be required for efficient RNA editing in genotype I [Casey et al., 1992; Zheng et al., 1992] is disrupted in Taiwan-3 isolate. This structure is also disrupted in genotype III (Peru-1) [Casey et al., 1993]. Thus, the editing structures of the three genotypes are different. Whether these differences will affect the editing efficiency is an interesting question. The sequence comparisons will help further determination of the molecular basis of these functions of delta antigen and RNA.

The sequence variations observed among the three genotypes might have resulted from mutations escaping from host immunity and may play some role in the biological and pathogenic differences of different genotypes of HDV. Another possible factor of HDV pathogenicity is the ratio of the large HDAg to the small HDAg [Govindarajan et al., 1993; Tang et al., 1994]. In our study, among five clones encoding HDAg in antigenomic RNA, three contain the stop codon (UAG), two contain UGG encoding tryptophan. A larger number of samples

are required to determine whether this ratio may differ in different genotypes.

ACKNOWLEDGMENTS

We thank Dr. M.M.C. Lai of University of Southern California for critical review and editorial assistance. We also thank Ms. C.S. Hwang and Ms. T.Y. Lin for excellent technical assistance. The study was supported by a research grant CMRP-326 from Chang Gung Memorial Hospital and Medical College to C.-M.L.

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